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HIF1 deubiquitination by USP8 is essential for ciliogenesis in normoxia

Albino Troilo, Irina Alexander, Sarah Muehl, Daniela Jaramillo, Klaus-Peter Knobeloch and Wilhelm Krek

Corresponding author: Wilhelm Krek, ETH Zurich

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Nonia Pariente

1st Editorial Decision 29 July 2013

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest, they also raise a number of concerns and feel the data should be strengthened before publication can be considered here.

Given that all referees provide constructive suggestions on how to make the work more conclusive and physiologically relevant, I would like to give you the opportunity to revise your manuscript. During revision, it would be particularly important to address the following issues:

- strengthening the evidence linking HIF1a and Rabaptin5 to ciliogenesis, by depletion and artificial activation, in normoxia and hypoxia

- reconciling the data regarding the amount of HIF1a expression in normoxia, as well as the effect of GFP alone on ciliogenesis in RPE-1 cells, with previous reports

- analyzing the USP8/HIF1/rabaptin5 pathway in ciliogenesis in more physiologically relevant cells

- analyzing the effect of USP8 on HIF2, which may be more relevant in a cancer setting

- tend to all other technical concerns raised by the referees

If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

The length of the current text is longer than we can accommodate, but I have noted that the end of the results and discussion section is an extended discussion, which seems rather redundant with the previous text and could be shortened. In addition, I would ask you to add subheadings to the results and discussion section, as this will help focus the article and give it the necessary structure. The introduction seems somewhat convoluted for the non-specialist and would benefit from editing.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 30,000 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

REFEREE REPORTS:

Referee #1:

In this manuscript Troilo et al. performed a screen searching for genes essential for ciliogenesis among 48 members of the USP family, where the cilia defect can be rescued by VHL depletion. In this screen they identified the ubiquitin-specific protease USP8. They show that USP8 acts as a HIF1a deubiquitinating enzyme. They further demonstrate that this property is linked to the VHL-dependency of USP8 as factor for ciliogenesis. USP8 counteracts the VHL-dependent ubiquitination of HIF1a, elevates the levels of HIF1a and maintains the repression of Rababtin 5 linking it to endosome trafficking and ciliogenesis.

This is a carefully performed study on an important topic of ciliary biology at the border between the pathogenesis of classical ciliopathies and cancer biology. I really like the concept of a regulatory circuit of USP8 and pVHL to fine tune the balance of HIF1a expression under normoxic conditions. The biochemical part of this paper is very strong. However, a number of critical issues should be addressed:

Major points:
1. A central point of this manuscript is the VHL-dependency of the essentiality of USP8 for ciliogenesis. The authors suggest the regulation of HIF1a and its downstream target Rabaptin5 as the mechanism which could explain the initially described effect of USP8/pVHL. However, they do not provide direct evidence that this is really the case. Therefore, it might be important to repeat the experiments in Fig. 1B/C with depletion of HIF and/or Rabaptin5, either by using the HIF-floxed-MEFs or RNAi. In addition: Do increased levels of HIF1a (by hypoxia or overexpression of a stabile version of HIF1a or treatment with cobald chlorid etc.) rescue the USP8-ciliogenesis-defect in VHL proficient cells? These experiments would strengthen the hypothesis, that HIF1a is the crucial target

of USP8 in this context.

2. Given the rather low efficiency of the USP8 knockdown and the dramatic effect on ciliogenesis, the effects demonstrated in Fig. 1B and 1C should be shown with at least one additional, independent shRNA against USP8 to exclude off-target effects (e.g. the ones used in Fig. 2A).

3. Is the observed effect relevant in VHL tumor cells?
In this study the authors used MEFs and RPE cells with RNAi. These are nice cell systems to study ciliogenesis and ciliary disassembly, however, these are not tumor cells. For VHL there are several VHL-/- carcinoma cell lines available. Is the observed ciliogenesis-defect of USP8 deficiency also rescued in such tumor cell lines carrying a VHL deletion and can this effect be modulated by re-expression of pVHL?

4. The authors performed gamma tubulin stainings in Fig. 1D. In the upper right panel the basalbody / centrosome appears as one single dot, as compared to the two distinct dots visible in the other pictures.
Is this just a problem of this one single depicted picture or do all cells with shRNA against USP8 look like this? If this is a more general observation, I would suggest to perform additional stainings for markers for mother and daughter centrioles to further describe this centrosomal phenotype. And how about centrosomes / basal bodies in HIF1a knockdown cells? This could be the mechanism explaining the defects in ciliogenesis.

5. What is the major subcellular localization of USP8? Is there a ciliary pool of this proteins, maybe at the ciliary base? Where does it interact with the HIF1a-pVHL complex?

Minor issues:
1. SEMs are missing in two data points of Fig. 1A.
2. The molecular weight marker should be added to Fig. 2 E/F/H/I.
3. In IF pictures the red color should be replaced by magenta with respect to color-blind readers (Fig. 1D/F, 3H, 4B/E).
4. I don't understand the sentence on pg. 5: "Hence, pVHL acts as a phenotypic suppressor of USP8 function in ciliogenesis". Shouldn't it rather be the "loss of VHL" which acts a suppressor?
5. Fig 1 E/F: Does Tamoxifen affect ciliogenesis in wildtype cells? This control should be added here.

Referee #2:

Troilo and colleagues perform a small siRNA screen of 48 USPs on the ciliary frequency of MEFs transfected with short hairpin shRNA knocking down either endogenous Vhlh or a negative control sequence. They identify that (1) USP8 deubiquitinates HIF1a; (2) that HIF1a regulates cilia, and (3) the Rab5 effector Rabaptin5 mediates the effects of these proteins on cilia through endosome trafficking.

Despite the three messages, which although all linked, make for a somewhat complexer story than EMBO Reports generally publishes, the paper is generally well crafted and has several novel components. The methods are appropriate and the results are reasonable and balanced.

The authors should consider the following:

1. HIF2a is entirely ignored in this study, yet this is increasingly considered to be relevant for the context of tumourigenesis. This reviewer would like to see at least one key experiment addressing the role of HIF2a in this context.
2. I am very surprised and skeptical by the high levels of HIF1a shown in normoxic HEK293 and RPE-1 cells (eg Fig 3C). The westerns are cut off and it is impossible to see a ladder (in the MEFs in Fig 3B such a ladder is shown). Is this HIF nuclear?
3. Fig. 3G: Many groups, many papers, have reported that expressing empty GFP vectors in RPE-1 cells reduces cilia frequencies significantly in these cells (this reviewer has never seen >55% ciliated RPE-1 cells with GFP). Yet Fig. 3G shows absolutely no effect of GFP alone on ciliation (>80%).

This finding requires further explanation because it deviates significantly from the collective data of many groups and perhaps this analysis should be extended?
4. The authors use MEFs and RPE-1 cells to examine the effects of USP8/HIF1a/VHL on ciliogenesis, yet 2 questions remain: Firstly, is it ciliogenesis they are scoring or ciliary maintenance? Secondly, neither of these cell types are relevant to ciliopathy phenotype observed in VHL patients... the authors should validate these effects in a cell line such as RPTECs.

Minor points:

1. It is not clear how the hits from the screen were validated, and whether the hits from the shVhlh screen (bottom panel of Fig1A) were validated at all. Also the hits in this screen are not further mentioned although several appear significant (eg USP10, Usp31, Usp47 etc). The authors should address these hits in the text.
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3. There is nothing "in vivo" about the overexpression cell studies labeled here as "in vivo".
4. Fig. 1C is not too convincing and is overexposed, especially since the tubulin levels seem to be considerably lower in the third lane.
5. How do the authors explain the HIF1a levels in Fig. 1H?
6. In their final paragraph, the authors touch upon the discussion between groups about whether the ciliary function of VHL is HIF-dependent or -independent, yet the authors sidestep their own papers, cell lines and conclusions. How do Krek and colleagues place these data in the context of their own experience and publications?

Referee #3:

In this manuscript, Troilo et al have investigated the genes which are essential for the formation of cilia and the loss of which can be compensated for by deficiency of pVHL. The authors identify the ubiquitin protease USP8 as such a gene and furthermore identify it as a HIF deubiquitinase which counteracts the ubiquitination of HIF by pVHL. This is a potentially important observation as it identifies both a mechanistic link between VHL deficiency and dysregulation of cilia and a new regulatory step in the hif pathway.

Comments:

1) the data in figure 1 demonstrates that USP8 is essential for cilial formation and that its loss can be compensated for by loss of pVHL suggesting a functional link between pVHL and USP. The authors then identify a HIF dependence of this event. However, these cells were cultured in Normoxia whereas numerous studies have shown that hif is normally not expressed in normoxic conditions. Why is HIF expressed in normoxia in these cells and how do the authors account for this? Are the authors proposing a functional role for HIF in Normoxia?

2) Using their cells, the author should demonstrate the effect of hypoxia on ciliogenesis? Furthermore, What is the impact of altering HIF levels by siRNA on ciliogenesis in hypoxia? These experiments would strengthen the link between HIF and cilia formation.

3) It appears in Fig 3A and B that almost total knockdown of HIF reduces the number of ciliated cells by 40 to 50% suggesting an alternative regulator. what is the effect of USP8 on HIF2 alpha? Is it effected in a similar way? This is important as HIF2 may by more important in cancer.

4) can the effects of USP8 on cilial formation be reproduced with a HIF activator such as DMOG. This would strengthen the evidence for a link between HIF and cilial formation. This experiment is required to more convincingly demonstrate a link between HIF and cilial formation.

5) can the authors demonstrate a general effect of USP on Hypoxic HIF activity using a HIF reporter?the authors should use such a system to strengthen the case for a counteracting system between VHL and usp8.

Point by point response to reviewer comments – EMBOR-2013-37688V1

Reviewer 1

This is a carefully performed study on an important topic of ciliary biology at the border between the pathogenesis of classical ciliopathies and cancer biology. I really like the concept of a regulatory circuit of USP8 and pVHL to fine tune the balance of HIF1a expression under normoxic conditions. The biochemical part of this paper is very strong. However, a number of critical issues should be addressed:

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To provide evidence for the existence of a direct link between the HIF1α and Rabaptin5 expression in primary cilia formation, we depleted HIF1α or Rabaptin5 alone or in combination in RPE-1 cells and scored for cilia. The experiment shows that the ciliogenesis defect observed upon loss of HIF1α was rescued by the additional depletion of Rabaptin5 (Fig. 3D). Also the large endosome phenotype induced by knockdown of HIF1α was rescued by co-depletion of Rabaptin5 (Fig. 3E). Similar results were obtained in MEFs (supplementary Fig. S6 online).

We have performed a series of new experiments addressing whether activation of HIF1α in USP8-depleted cells would reverse the associated ciliogenesis defect. We stabilized HIF1α in RPE-1 cells through different means including hypoxia (supplementary Fig. S8A online), treatment of cells with prolyl hydroxylase inhibitor (supplementary Fig. S8B online) or overexpression of a stable mutant form of HIF1α that lacks the ODD domain (HIF1α-ΔODD) (supplementary Fig. S8C online), and observed in each case a resue of the ciliogenesis defect induced by knockdown of USP8. Even the ciliogenesis defect caused by depletion of HIF1α in normoxia was rescued when cells were placed in hypoxia (supplementary Fig. S8D online), due to increased expression of the remaining HIF1α (which reached levels similar to those seen in normoxic cells) that originates from the incomplete depletion by HIF1α-targeting shRNAs.

Together, these data strengthen the evidence linking HIF1α and Rabaptin5 to the process of ciliogenesis. Moreover, they suggest that HIF1α is a crucial target of USP8 in the context of primary cilia formation.

2. Given the rather low efficiency of the USP8 knockdown and the dramatic effect on ciliogenesis, the effects demonstrated in Fig. 1B and 1C should be shown with at least one additional, independent shRNA against USP8 to exclude off-target effects (e.g. the ones used in Fig. 2A).

We knocked-down mouse Usp8 and human USP8 in MEFs and RPE-1 cells,

respectively, using corresponding second shRNAs. The experiments confirm the observed effect on ciliogenesis (supplementary Fig. S1C, D online). We wish to point out that genetic deletion of Usp8 in MEFs derived from Usp8 floced mice likewise negatively affects ciliogenesis (Fig 1E, F), argueing against an off-target effect.

3. Is the observed effect relevant in VHL tumor cells? In this study the authors used MEFs and RPE cells with RNAi. These are nice cell systems to study ciliogenesis and ciliary disassembly, however, these are not tumor cells. For VHL there are several VHL-/- carcinoma cell lines available. Is the observed ciliogenesis-defect of USP8 deficiency also rescued in such tumor cell lines carrying a VHL deletion and can this effect be modulated by re-expression of pVHL?

To address this point we performed cilia formation experiments in the *VHL*-deficient renal cell carcinoma cell line RCC4 and a corresponding isogenic counterpart that had been engineered to re-express wild-type pVHL. Depletion of USP8 in the RCC4 (VHL-/-) cells had little, if any, effect on cilia formation, while it affected cilia formation in pVHL-reconstituted RCC4 cells (supplementary Fig. S8E online). These results underscore the pVHL-dependency of USP8 function in ciliogenesis in renal carcinoma cells.

4. The authors performed gamma tubulin stainings in Fig. 1D. In the upper right panel the basalbody / centrosome appears as one single dot, as compared to the two distinct dots visible in the other pictures. Is this just a problem of this one single depicted picture or do all cells with shRNA against USP8 look like this? If this is a more general observation, I would suggest to perform additional stainings for markers for mother and daughter centrioles to further describe this centrosomal phenotype. And how about centrosomes / basal bodies in HIF1a knockdown cells? This could be the mechanism explaining the defects in ciliogenesis.

This is indeed only an issue in the picture portrayed in Fig. 1D. Examination of our basal bodies stainings revealed no quantitative differences between non-silencing and USP8 or HIF1α knock down cells. Therefore, we replaced the original picture with a new picture that reflects this fact and shows to distinct dots.

5. What is the major subcellular localization of USP8? Is there a ciliary pool of this proteins, maybe at the ciliary base? Where does it interact with the HIF1a-pVHL complex?

Several studies revealed that USP8 is a cytoplasmic and membrane protein (Mizuno E. et al, 2005, *Molecular Biology of the Cell*, Row P. E. et al, 2006, *The Journal of Biological Chemistry*, Niendorf S. et al, 2007, *Molecular and Cellular Biology*). It may be difficult to assess where USP8 interacts with the HIF1α-pVHL complex as these interactions are likely highly dynamic in particular in normoxia. A cell biological study addressing USP8 localization and dynamics in the context of cilia formation and resorption would certainly be warranted but it is in our opinion beyond the scope of this manuscript.

Minor issues:

1. SEMs are missing in two data points of Fig. 1A.

The SEMs were added according to the reviewer's suggestion.

2. The molecular weight marker should be added to Fig. 2 E/F/H/I.

Molecular weight markers were added according to the reviewer's suggestion except for Fig. 2E we believe it is not needed.

3. In IF pictures the red color should be replaced by magenta with respect to colorblind readers (Fig. 1D/F, 3H, 4B/E).

The red colour was replaced in the IF pictures according to the reviewer's suggestion.

4. I don't understand the sentence on pg. 5: "Hence, pVHL acts as a phenotypic suppressor of USP8 function in ciliogenesis". Shouldn't it rather be the "loss of VHL" which acts a suppressor?

It is the loss of pVHL that results in a phenotypic suppression of USP8 function in ciliogenesis. This has been corrected.

5. Fig 1 E/F: Does Tamoxifen affect ciliogenesis in wildtype cells? This control should be added here.

To test whether Tamoxifen affects ciliogenesis, we treated wild-type MEFs with 4-hydroxytamoxifen (4-OHT) for 8 day. The last two days of treatment cells were serum starved to induce ciliogenesis. Neither USP8 levels nor ciliogenesis is affected by tamoxifen (supplementary Fig. S1E online).

Reviewer 2

Despite the three messages, which although all linked, make for a somewhat complexer story than EMBO Reports generally publishes, the paper is generally well crafted and has several novel components. The methods are appropriate and the results are reasonable and balanced. The authors should consider the following:

1. HIF2a is entirely ignored in this study, yet this is increasingly considered to be relevant for the context of tumourigenesis. This reviewer would like to see at least one key experiment addressing the role of HIF2a in this context.

We have performed several experiments addressing the role of HIF2α in the context of ciliogenesis. First, we downregulated HIF2α alone or in combination with HIF1α in RPE-1 cells. In this cell type, we observed that depletion of HIF2α has only a minor effect on ciliogenesis either when depleted alone or in combination with HIF1α (supplementary Fig. S5A online). qRT-PCR analysis of endogenous mRNA levels of HIF2α and HIF1α in RPE-1 cells revealed that HIF2α mRNA levels are about 7-fold lower than mRNA levels of HIF1α (supplementary Fig. S5B online). This difference in expression level may contribute, in part, to the differential effects of HIF2α and HIF1α depletion in the context of RPE-1 cells. HIF2α and HIF1α have context-dependent functions, as was previously reported (Gordan J. D. and Simon M. C., 2007, *Current Opinion in Genetics and Development*). It is therefore entirely possible that in other cell types, HIF2α may contribute to a greater extend to ciliogenesis as HIF1α. Consistent with such a view, we found that depletion of USP8 in HEK293T cells reduced protein levels of both HIF1α and HIF2α (supplementary Fig. S3D online). Moreover, a stable proline-mutant derivative of HIF2α when overexpressed in HEK293T cells, coimmunoprecipitated with endogenous USP8 (supplementary Fig. S3C online). Finally, consistent with the finding that the PAS domain of HIF1α is necessary and sufficient for interactions with USP8, we also observed binding of HIF2α to GST-USP8 *in vitro* (supplementary Fig. S3B online).

2. I am very surprised and skeptical by the high levels of HIF1a shown in normoxic HEK293 and RPE-1 cells (eg Fig 3C). The westerns are cut off and it is impossible to see a ladder (in the MEFs in Fig 3B such a ladder is shown). Is this HIF nuclear?

In the experiments shown in Figs 2A, B, whole cell lysates of HEK293T cells were processed for Western blot. HIF1α levels appear as single bands because the lysates were resolved on a 10% SDS-gel using a mouse mAb antibody from BD-Transduction lab as indicated in the figure legends and described in materials methods. In Fig. 3C, as in all other experiments, the whole cell lysates for HIF1α Western blotting were resolved on an 8% SDS-gel and blotted with an anti-rabbit HIF1α antibody from Novus Biologicals.
We replaced the HIF1α Western blot displayed in Fig. 3C of the original version with a lower exposure of the very same Western blot (new Fig. 3D of the revised version). In the lower exposure of the Western blot, the ladder is clearly visible.

There are several publications that show that HIF1α is clearly detectable in multiple cell types under normoxic conditions (Lumm J. J. et al, 2007, *Gens and Development*, Mills C. N. et al, 2009, *Molecular Cancer*, Cicchillitti L. et al, 2012, *The Journal of Biological Chemistry*). Given this evidence, we assume that under normoxia, HIF1α is, at least in part, in the nucleus to provide basal expression of certain HIF target genes such as CA9 (see supplementary Fig. S2C online).

3. Fig. 3G: Many groups, many papers, have reported that expressing empty GFP vectors in RPE-1 cells reduces cilia frequencies significantly in these cells (this reviewer has never seen >55% ciliated RPE-1 cells with GFP). Yet Fig. 3G shows absolutely no effect of GFP alone on ciliation (>80%). This finding requires further explanation because it deviates significantly from the collective data of many groups and perhaps this analysis should be extended?

We appreciate this reviewer's comment. We have carefully analysed again our row data and we did not observe much effect of GFP expression on cilia formation frequency. The number of ciliated RPE-1 cells transfected with an empty pEGFP vector was repeatedly close to 80% (supplementary Fig. S9A online). To illustrate this point, we display images of RPE-1 cells expressing different levels of GFP protein that were stained with an acetyl-tubulin antibody. As shown in supplementary Fig. S9A online, irrespective of GFP expression levels the majority of cells display primary cilia. As transfection reagent for RPE-1 cells we always use the 'SE Cell Line 96-well Nucleofector™ Kit' (Lonza). This is an electroporation-based technique that allows to transfect RPE-1 cells with high efficiency without affecting cell viability. In addition, the cell batch of RPE-1 we use in our experiments may be another source of difference. Finally and importantly, we are not the only group that observes high frequency of ciliated GFP-transfected RPE-1 cells. A recently published paper also reported on RPE-1 cells that were transfected with a GFP empty vector and displayed cilia in more than 80% of the cases (Kuhns S. et al, 2013, *The Journal of Cell Biology*).

4. The authors use MEFs and RPE-1 cells to examine the effects of USP8/HIF1a/VHL on ciliogenesis, yet 2 questions remain: Firstly, is it ciliogenesis they are scoring or ciliary maintenance? Secondly, neither of these cell types are relevant to ciliopathy phenotype observed in VHL patients... the authors should validate these effects in a cell line such as RPTECs.

We score in this work for cilia formation (ciliogenesis) and not cilia maintenance, which is critical after the primary cilium is formed and can be reverted by the addition of growth factors. To address the point of the reviewer, we performed our ciliogenesis experiments additionally in RPTECs. As shown in Fig. 3C, depletion of USP8 or HIF1α in RPTECs impaired ciliogenesis, thus further corroborating the results obtained in other cell systems.

Minor points:

1. It is not clear how the hits from the screen were validated, and whether the hits from the shVhlh screen (bottom panel of Fig1A) were validated at all. Also the hits in this screen are not further mentioned although several appear significant (eg USP10, Usp31, Usp47 etc). The authors should address these hits in the text.

The primary screen was performed three times (three biological replicates) in MEF shCtr and the hits were tested again in terms of their requirement for ciliogenesis (see supplementary Fig S1A, B online). We were able to confirm a requirement for Usp8 and Usp39. The counterscreen in MEF shVhl was performed once with three technical replicates. This counterscreen revealed among various hits Usp8. Because Usp8 fulfilled the original quest to identify USPs required for ciliogenesis only in VHL-proficient but not deficient cells, we focused out further analysis on this specific Usp.

2. The nomenclature for the murine orthologues is not accurate in any of the MEF experiments.

The nomenclature for the murine orthologous was changed as suggested by the reviewer.

3. There is nothing "in vivo" about the overexpression cell studies labeled here as "in vivo".

We removed the word *in vivo* as suggested by the reviewer.

4. Fig. 1C is not too convincing and is overexposed, especially since the tubulin levels seem to be considerably lower in the third lane.

Figure 1C displays Western blot data for USP8, pVHL and tubulin. In this panel, tubulin is expressed at equal levels.

5. How do the authors explain the HIF1a levels in Fig. 1H?

We thank the reviewer for pointing this out. We assume that the reviewer refers to Fig. 2H. We have now clarified this point in the revised version. Although in all 5 lanes of Fig. 2H, we transfected the identical amounts of HIF1α expression plasmid, lanes 3-5 contain in addition other plasmids as indicated. Thus, in the first two lanes, less total plasmid was in the transfection mix, which is likely the reason for higher expression of HIF1α. The first two lanes (where the same amount of total plasmid was transfected) represent a control for the specificity of the HIF1α immunoprecipitation (no HIF1α protein is seen in the IgG control). In the other 3 lanes (lanes 3-5), where USP8 wild-type and mutant plasmids were added in addition to a HA-Ub plasmid, the identical amounts of total plasmid were present and as seen on the Western blot, very similar levels of HIF1α were detected in the immunoprecipitates in all three lanes (that is lanes 3-5).

6. In their final paragraph, the authors touch upon the discussion between groups about whether the ciliary function of VHL is HIF-dependent or -independent, yet the authors sidestep their own papers, cell lines and conclusions. How do Krek and colleagues place these data in the context of their own experience and publications?

We have now addressed this issue in the discussion part and included a paragraph to better describe the differences in the approach and cell types used in the various studies in the past and connect this to the current findings. As mentioned in the discussion, previous studies conducted in various cell contexts revealed different effects of oxygenpathway components on ciliary dynamics. Studies conducted in primary cells, such as RPTECs or MEFs, demonstrate that depletion of pVHL does not affect ciliogenesis. However, pVHL-deficiency sensitized cells for precocious cilia disassembly in response to growth factor cues, which has been mechanistically linked to loss of pVHL's microtubule stabilization properties and resultant destabilization of the ciliary axoneme

(Thoma et al., 2007, *Nature Cell Biology*). Consistent with this cell biological evidence, kidney-specific loss of *Vhl* alone is insufficient for early onset of cyst formation (Rankin E. B. et al, 2006, *Cancer Research*). Rather rapid onset of cyst formation and loss of primary cilia *in vivo* requires cooperation of Vhl nullizygosity with Pten loss (Frew I. J. et al, 2008, *EMBO J*).

VHL-deficient renal carcinoma cells display different behaviors with respect to their ability to form primary cilia in response to reexpression of pVHL and/or HIFα subunits insofar as in some cells pVHL-induced ciliogenesis is HIF-independent (Lutz M. S. and Burk R. D., 2006, *Cancer Research*) and in others HIF-dependent (Esteban et al., 2006, *JASN*). This difference might be explained by the different spectra of mutations affecting proto-oncogenes and tumor suppressor genes present in these cell lines. Indeed, in our own experience, VHL-negative ccRCC cell lines display major differences with respect to their capacity to form cilia. Some fail entirely to form cilia while others do so with high frequency (A. T. and W. K., unpublished). In the context of our findings described here, hypoxia or loss-of-function mutations of VHL commonly found in ccRCC will supersede a requirement for USP8 in HIF1α abundance control. Accordingly, the pVHL–USP8–HIF1α regulatory circuitry is expected to operate primarly in VHL-proficient cells under normoxic conditions to fine-tune HIF1α levels and transcriptional output.

Reviewer 3

In this manuscript, Troilo et al have investigated the genes which are essential for the formation of cilia and the loss of which can be compensated for by deficiency of pVHL. The authors identify the ubiquitin protease USP8 as such a gene and furthermore identify it as a HIF deubiquitinase which counteracts the ubiquitination of HIF by pVHL. This is a potentially important observation as it identifies both a mechanistic link between VHL deficiency and dysregulation of cilia and a new regulatory step in the hif pathway.

Comments:

1. The data in figure 1 demonstrates that USP8 is essential for cilial formation and that its loss can be compensated for by loss of pVHL suggesting a functional link between pVHL and USP. The authors then identify a HIF dependence of this event. However, these cells were cultured in Normoxia whereas numerous studies have shown that hif is normally not expressed in normoxic conditions. Why is HIF expressed in normoxia in these cells and how do the authors account for this? Are the authors proposing a functional role for HIF in Normoxia?

A main finding of our study is to propose a normoxic function of HIF1α in the contest of ciliogenesis and provide an underlying mechanism. Despite its well-characterized hypoxic function, several reports have shown that HIF1α is expressed in normoxic conditions (Lumm J. J. et al, 2007, *Gens and Development*, Mills C. N. et al, 2009, *Molecular Cancer*, Cicchillitti L. et al, 2012, *The Journal of Biological Chemistry*) and have described potential roles in this setting (Lumm J. J. et al, 2007, *Gens and Development*, Mills C. N. et al, 2009, *Molecular Cancer*, Cicchillitti L. et al, 2012, *The Journal of Biological Chemistry*). In addition, consistent with the fact that HIF1α levels is controlled at multiple levels including translation, HIF1α levels can be influenced in normoxic conditions by PI3K-mTORC1 signaling (Zhong H. et al, 2000, *Cancer Research*, Hudson C. C. et al, 2002, *Molecular and Cellular Biology*).

2. Using their cells, the author should demonstrate the effect of hypoxia on ciliogenesis? Furthermore, What is the impact of altering HIF levels by siRNA on ciliogenesis in hypoxia? These experiments would strengthen the link between HIF and cilia formation.

We have depleted HIF1α in RPE-1 cells and assessed whether the ciliogenesis defect observed can be rescued when cells are placed in hypoxia. This was indeed the case (supplementary Fig. S8D online). This rescue can be understood in light of the fact that due to incomplete depletion of HIF1α by HIF1α-targeting shRNAs, the levels fo the remaining protein increased in hypoxia reaching levels similar to those seen in normoxic cells (supplementary Fig. S8D online).

3. It appears in Fig 3A and B that almost total knockdown of HIF reduces the number of ciliated cells by 40 to 50% suggesting an alternative regulator. what is the effect of USP8 on HIF2 alpha? Is it affected in a similar way? This is important as HIF2 may by more important in cancer.

We have performed several experiments addressing the role of HIF2α in the context of ciliogenesis. First, we downregulated HIF2α alone or in combination with HIF1α in RPE-1 cells. In this cell type, we observed that depletion of HIF2α has only a minor effect on ciliogenesis either when depleted alone or in combination with HIF1α (supplementary Fig. S5A online). qRT-PCR analysis of endogenous mRNA levels of HIF2α and HIF1α in RPE-1 cells revealed that HIF2α mRNA levels are about 7-fold lower than mRNA levels of HIF1α (supplementary Fig. S5B online). This difference in expression level may contribute, in part, to the differential effects of HIF2α and HIF1α depletion in the context of RPE-1 cells. We note however, that HIF2α and HIF1α have context-dependent functions, as was previously reported (Gordan J. D. and Simon M. C., 2007, *Current Opinion in Genetics and Development*). It is therefore entirely possible that in other cell types, HIF2α may contribute to a greater extend to ciliogenesis as HIF1α. Consistent with such a view, we found that depletion of USP8 in HEK293T cells reduced protein levels of both HIF1α and HIF2α (supplementary Fig. S3D online). Moreover, a stable proline-mutant derivative of HIF2α when overexpressed in HEK293T cells, coimmunoprecipitated with endogenous USP8 (supplementary Figs S3C online). Finally, consistent with the finding that the PAS domain of HIF1α is necessary and sufficient for interactions with USP8, we also observed binding of HIF2α to GST-USP8 *in vitro* (supplementary Fig. S3B online).

4. can the effects of USP8 on cilia formation be reproduced with a HIF activator such as DMOG. This would strengthen the evidence for a link between HIF and cilial formation. This experiment is required to more convincingly demonstrate a link between HIF and cilial formation.

We have performed a series of experiments to strengthen the link between HIF1α and ciliogenesis. We stabilized HIF1α in RPE-1 cells through different means including hypoxia (supplementary Fig. S8A online), treatment of cells with prolyl hydroxylase inhibitor (supplementary Fig. S8B online) or overexpression of a stable mutant form of HIF1α that lacks the ODD domain (HIF1α-ΔODD) (supplementary Fig. S8C online), and observed in each case a resue of the ciliogenesis defect induced by knockdown of USP8.

5. can the authors demonstrate a general effect of USP on Hypoxic HIF activity using a HIF reporter? the authors should use such a system to strengthen the case for a counteracting system between VHL and usp8.

To address this point we depleted USP8 or HIF1α in RPE-1 cells and then transfected these cells with a HIF(CXCR4)-promoter – luciferase reporter plasmid. These cells were then either kept under normoxic conditions or placed under hypoxia. Under both conditions, reporter activity was significantly reduced in USP8- or HIF1α-depleted cells (supplementary Fig. S2B online). This is also consistent with Western blot data demonstrating reduced levels of HIF1α under hypoxia conditions when USP8 was depleted (supplementary Fig S8A). In addition, qPCR analysis revealed that the mRNA levels of endogenous CA9, a well-established HIF target (Wykoff C. C. et al. 2000,

Cancer Research), is significantly reduced in cells depleted for USP8 or HIF1α (supplementary Fig. S2C online). Together these data support the notion that the counteracting activities of pVHL and USP8 regulate HIF1α levels.

2nd Editorial Decision 14 October 2013

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- The manuscript length exceeds what we can accommodate; please shorten the main text to a maximum of 30,000 characters, including spaces. Shortening may be made easier by combining the Results and Discussion into a single section, which we require, and which will help eliminate the redundancy that is inevitable when discussing the same experiments twice. In addition, although basic Materials and Methods required for understanding the experiments performed must remain in the main text, additional detailed information may be included as Supplementary Material.

- I have noted that the legends to figures 2A, 2B and supplementary figure 6 lack information regarding the number of independent experiments performed and the identity of the error bars. In addition, figures 3D-F, 1A and Supp 9B were performed one or two independent times. Please note that, in these cases, it is incorrect to calculate errors (for guidance, please refer to: Cumming et al. JCB 2007). Please either increase the number of independent experiments represented to a minimum of 3 (I appreciate this is not possible for Fig1A) or, alternatively, plot the individual data points obtained.

- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited versions below my signature and let me know if you do NOT agree with any of the changes. In addition, I have a query in a place that I am unsure of having understood your meaning.

- Lastly, we now encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

Once all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

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Edited title and abstract

HIF1α deubiquitination by USP8 is essential for ciliogenesis in normoxia

Loss of primary cilia is a key feature of von Hippel-Lindau tumor suppressor (VHL)- associated pathology. Although VHL-deficiency predisposes cells to precipitous cilia disassembly in response to growth factor cues [OK?], it does not affect ciliogenesis. Here, using a siRNA-based screen to find genes that are essential for ciliogenesis only in the presence of VHL, we identify ubiquitin-specific protease (USP)8. The pVHL-dependency of USP8 for ciliogenesis is directly linked to its function as a HIF1α deubiquitinating enzyme. By counteracting pVHL-mediated ubiquitination of HIF1α, USP8 maintains a basal expression of HIF1α and HIF transcriptional output in normoxia, including the repression of Rabaptin5, which is essential for endosome trafficking-mediated ciliogenesis.

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REFEREE REPORTS:

Referee #1 (Report):

The quality of the manuscript increased dramatically in this revised version. With the newly added data all of my concerns were addressed in a very convincing way. Therefore, I believe that this manuscript is highly suitable for publication.

Referee #2 (Report):

The authors have improved the manuscript sufficiently with the revisions made.

Referee #3 (Report):

The authors have mostly addressed my concerns. However, i remain very surprised by the reported role of hif in normoxia. The authors refer to some previous papers relating to effects of hif in normoxia but there are many many more papers in which the functional effects of hif are evident only in hypoxia. This is especially surprising since there is no effect of hypoxia or hydroxylase inhibition on ciliogenesis as reported in the new experiments. I am surprised by this. Therefore, the fact that the authors are reporting a hypoxia-independent role for hif which is evident in normoxia should be reflected in the title by (for example) the addition of the words 'in normoxia' to the end of the current title.

2nd Revision - authors' response 25 October 2013

Thank you very much for the prompt review of our revised manuscript. Please find enclosed a revised version of the manuscript that contains all requested changes including full scans of the key immunoblots shown in the manuscript and the original source data behind the graphs. We also agree with your proposal of the title and the changes in the abstract.

We are grateful to your efforts and look forward to hearing from you.

3rd Editorial Decision 30 October 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

We will send you the license to publish forms and page charge authorization form with further instructions, in a separate e-mail, in a few days. Thank you for your patience.

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